THE ORIGIN OF BACTERIAL SPECIES

Genetic Recombination and Factors Limiting It Between Bacterial Populations

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I. Introduction

The centenary year of the publication of *The Origin of Species* has drawn to a close, and the bacteriologist realizes that he has been somewhat overlooked in the celebration of Darwin's important contribution to scientific and intellectual progress. Yet this neglect of the bacteriologist is undeserved, for as this review will try to show, recent discoveries in bacterial genetics have revealed that the Darwinian view of evolution encompasses not only the world of "higher" plants and animals, but the world of bacteria as well. Heretofore the principal difficulty in integrating the bacteria within the Darwinian model of evolution was the apparently different ways of defining species for sexually reproducing, "higher" plants and animals, on the one hand, and for clonally reproducing, acellular organisms, on the other. This difficulty, however, is largely overcome by recognizing that bacteria are indeed capable of transferring and recombining genetic material, which is accomplished through biparental, sexual reproduction in "higher" organisms, and by defining species universally in terms of the capacity to exchange genetic material.

The plan of this article is to review current concepts of speciation, to show how our knowledge of genetic recombination in bacteria affords a definition of bacterial species in genetic terms, and then finally to consider the usefulness of such a view of bacterial species for our understanding of bacterial evolution.

II. The Species Concept

The Darwinian hypothesis proposed that species of plants and animals are the products principally of two forces: the origin of heritable variations in individual organisms, and the selection of those variations endowing their bearers with advantage of one kind or another over natural competitors for survival and reproduction (18). The science of genetics, which arose and has undergone rapid development since the days of Darwin, has revealed the nature of the genetic alterations, or mutations, which serve as the raw materials of selection: their low frequency under natural conditions, and their randomness, that is, the lack of correspondence between the selective advantage of the mutant phenotype and the environment in which the mutations are produced. We have learned also

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of the important role that sexual reproduction plays in increasing the rate of production of genetic variety in a population, through bringing together, by recombination, different mutations that arise in different individuals.

The high incidence of an adaptive genetic constitution within a species is the result, then, of spontaneous mutation, recombination, and selection. However, once an adaptive genotype (or, more properly speaking, a set of adaptive genotypes) is acquired by a population, are there any mechanisms to prevent its rapid breakdown by the "blind" forces of mutation and recombination? For it is obvious that mutation and recombination can just as efficiently bring nonadaptive diversity to a population as they can adaptive diversity. Of course, selection could continue to weed out adverse or deleterious mutations, but again thanks to the investigations of geneticists, we know that special genetic mechanisms may arise to reinforce the work of selection. These mechanisms, which are themselves the product of mutation and recombination, serve to limit the exchange of genetic material by members of a given population. Recombination becomes effectively limited to those individuals sharing a common adaptive gene pool. Thus, such mechanisms have been termed *isolating mechanisms* (21).

There are a number of ways in which two populations of sexually reproducing organisms can become genetically isolated from each other. Sperm-egg interactions can become highly specific, so that sperm of one population are incapable of fertilizing the eggs of the other population. Copulatory organs can become so specific, that the male genitalia of one population are mechanically incompatible with the female genitalia of the other. Complex breeding behavior, involving the precopulatory antics of male-female pairs, may also become highly specific. In plants, flowers may be produced at different times in different species, and so on.

This brief review of our knowledge of speciation in higher plants and animals has been set forth to see if we can find similar processes available and utilized by bacteria. It is perhaps important to distinguish between availability and utilization, for as will be pointed out later, good evidence exists that similar processes are available to the bacteria, although the evidence is less certain that they are actually utilized in nature.

We might properly begin by inquiring what a species is in the world of clonally reproducing bacteria. We know how this problem has been handled for the higher plants and animals, which reproduce sexually. Long before geneticists began to speak of isolating mechanisms, taxonomists developed rules of classification according to which species were defined. In general, one may say that a population of individuals sharing a particular constellation of ecological, physiological, morphological, and behavioral characteristics was called a species. The particular constellation of characteristics adopted was one that was sufficient to demarcate a given population from others, and that was especially stable, that is, was reproducible in a given environmental situation and did not change appreciably from one generation to the next. Today the geneticist would refer to a species as a population of potentially interbreeding individuals, that is, one in which genetic recombination can occur between any pair of its members but is prevented between its members and those of other species populations. In general, it is probably true that the taxonomist's species corresponds very closely with the geneticist's species. But this is not always the case. Some populations, regarded for good and compelling reasons by the taxonomist as different species, may be found to be capable of interbreeding. The solution to this problem has usually been one of two types:

a. If the two populations are separated by a sufficiently large geographical distance or barrier, it is possible to assume that they descended from some common ancestral species, that migration separated them, that mutation, recombination, and selection differentiated them for their ecological specialties, and that a mechanism isolating them genetically has not arisen because it has no selective advantage except in an area where, or at a time when the two populations confront each other. The two populations can thus be considered subspecies or races, as you will. This is not to say that all geographically separated populations descended from a common ancestral group are capable of interbreeding. It is possible that some such groups acquired, possibly as a secondary consequence of other morphological and physiological differentiation, an effective mechanism isolating them reproductively. Such groups are usually termed *allopatric* species (see (56)).

b. If the two populations are not separated geographically, one may assume again that they are the descendants of a common ancestral population, that mutation and recombination are differentiating them, and that unless the advantage to be gained by an isolating mechanism (*i.e.*, by preservation of the differentiation) outweighs whatever adaptive advantage there may result from genetic flow between them, the two populations will remain nothing more than two samples drawn from a larger, heterogeneous population. On the contrary, if an isolating mechanism is of relative advantage, as in the case of populations adapting to different ecological niches, and if it is actually beginning to operate, the two populations may be regarded as incipient species.

The problem of defining species among bacteria would, of course, be fundamentally different if no exchange of genetic material ever occurred between individual bacteria. We are just beginning to know something about these methods of genetic exchange, and so it is no surprise that the convention of naming species in bacteria is similar to that of the classic taxonomist in naming species of higher plants and animals. One chooses, to distinguish a bacterial species, a particular constellation of ecological, morphological, physiological, and biochemical characteristics that is unique and stable enough to demarcate it from other species, for which other constellations of characteristics are found. It will be interesting to see how well the species of the bacterial taxonomist accord with whatever species the bacterial geneticist arrives at by utilizing the criterion of potential exchange of genetic material.

Before doing so, one must consider what we know of bacterial mutation and recombination.

III. Bacterial Mutation and Recombination

A. Mutation

The bacteriologist rarely deals with the individual bacterium. Rather he is nearly always working with a culture, or population, consisting of several millions or billions of individuals, which, alas, he has often been wont in the past to regard as a homogeneous collection and hence as a unit. Thus, when bacterial populations were found to undergo genetic changes under varying environmental conditions, the view developed that bacteria, unlike the "higher" organisms, were genetically flexible and could adapt their genotype fairly readily to the exigencies of the environment (30).

It was not until the advent of the bacterial geneticist that this view could be disproved. For the bacterial geneticist employed methods to discriminate between what was going on at the level of the individual bacterium and what was going on in the culture or population as a whole. Suffice it to say, his methods were able to establish that bacteria are no different from "higher" organisms in their mutability. Genetic changes are rare spontaneously, but when they do occur there is no necessary correspondence between the change and the kind of environment in which it appears. Thanks, however, to these random mutations, a culture is always sufficiently heterogeneous that when the environment changes, mutants adapted to the new environment are selected. As Stanier (79) has aptly pointed out, the haploid condition of bacteria (42) causes newly arising mutations to be exposed immediately to the action of natural selection, thus enhancing the rapidity with which selection can cull the genotypes within a population. The roles that mutation and selection play in the survival of a bacterial population living in a fluctuating environment are probably very important.

The first evidence that bacterial mutations occur at random was statistical in nature (40, 54, 60). The argument was that, if mutations occur rarely (less than once per several generations) and at random, then—in the absence of environmental conditions favoring the reproduction of the mutants—very large fluctuations would be expected between the mean numbers of mutants appearing in parallel cultures inoculated with equal but small numbers of bacteria drawn from a common population. On the other hand, large fluctuations would not be expected if direct genetic adaptation accounted for the appearance of mutations. In the latter case, a bacterium may have a small probability of adapting genetically to a given environment, but, in any case, the number of adapted mutants appearing in a culture should be dependent only upon the total number of bacteria in that culture. Suffice it to say that the prediction of the hypothesis of rare, random mutation was fulfilled in all cases adequately studied [for

example, bacterial resistance to virus (54, 59); bacterial resistance to drugs and antibiotics (61, 77, 83); bacterial ability to ferment sugars (71)].

An elegant proof of the randomness of bacterial mutations was provided by the Lederbergs (46). This proof is independent of statistical considerations and requires only the simplicity of replica plating. By this method a large number of cells from a bacterial population is plated on the surface of a nonselective medium (*i.e.*, one in which the mutants do not have a selective advantage) and after a suitable period of incubation replica plates are made by transferring impressions of the superficial growth to another plate by means of a velvet disc (or other similar device). The replica plate contains a selective medium that permits only mutants of the desired type to grow. By comparing the original with the replica plate, one can locate those colonies on nonselective medium from which the bacteria capable of growing on the selective medium were drawn. Only the hypothesis of random spontaneous mutations predicts that these colonies already contain mutant clones, which may be large or small in size depending upon how early or late, respectively, the spontaneous mutations appeared in the growth of the colonies. By isolating these colonies and plating again on nonselective medium, one should find by replica plating on selective medium that the proportion of mutant cells is greatly increased. In a series of isolations, platings, and replica tests, one should finally obtain a "pure" culture of mutant cells. This finding was obtained for a number of cases of bacterial mutation (46, 77, 83).

Either the statistical fluctuation test or the indirect selection method provides an estimate of the frequency of spontaneous mutations in a given population. Generally, these frequencies are low. Expressed as the probability of mutation per bacterium per generation, they vary usually from about 10^{-7} to 10^{-10}. It is clear, therefore, that bacterial mutations are rare events and occur in the absence of conditions which specifically favor the mutants. The bacteria represent no exception to the general rule observed among "higher" plants and animals that inherited variations occur at random.

B. Recombination

Given the inherited variations provided by mutation, the amount of genetic variety possible in a population can be increased by combining, in the various possible ways, mutated genetic material of different individuals. Such increase in genetic variety is generally provided by recombination. Recombination has been defined recently by Pontecorvo (64) in a useful way. He considers it ". . . any process which gives origin to cells or individuals associating in new ways two or more hereditary determinants in which their ancestors differed: for instance, cells with determinants *Ab* or *aB* descending from other cells with *AB* or *ab*." Here *A* and *a*, or *B* and *b*, refer to the alternative states of a given hereditary determinant affecting a particular property of the cell or individual. Thus, *A* may symbolize the determinant in a bacterium endowing it with streptomycin resistance, while *a* symbolizes the alternative, or homologous, determinant making the bacterium sensitive to streptomycin; *B*, on the other hand, may refer to the determinant making the bacterium capable of fermenting a specific sugar, while *b* refers to its homologue rendering the bacterium unable to ferment this sugar.

In the case of bacteria, genetic recombination operates in the following way. From a particular kind of action occurring between two populations, one that is genetically *AbCd*. . . . , and one that is genetically *aBcD*. . . . , can be derived other populations which possess some or all of the other possible combinations: *ABCD*. . . . , *abcd*. . . . , *ABCd*. . . . , *abcD*. . . . , *AbcD*. . . . , *aBCd*. . . . , *Abcd*. . . . , *aBCD*. . . . , *ABcd*. . . . , *abCD*. . . . , *ABcD*. . . . , *abCd*. . . . , *AbCD*. . . . , and *aBcd*. . . . . The fact of genetic recombination demonstrates that the hereditary material of bacteria, like that of other organisms, consists of separable elements, or genes, which determine different specific functions. These separable determinants are not necessarily exchanged independently of each other during recombination. Two or more determinants may be exchanged in a block, and the frequency of such block exchanges indicates the degree of linkage between any pair or set of genes. A number of lines of evidence (20, 41) point strongly to the likelihood that the genes of bacteria are organized in linear arrays. In some cases, the bacterial genes can be mapped with considerable precision.

The significance that recombination may have in bacterial evolution lies in the fact that there are generally several genetic differences between any two recognized species. Each species-dis-

tinguishing characteristic is usually capable of mutation independently of the others. Two such fairly similar species as *Aerobacter aerogenes* and *Escherichia coli* differ in their ability to produce indole from tryptophan, to attack citrate as a sole energy source for growth, to produce acetylmethylcarbinol during glycolysis, and to accumulate organic acids in sugar fermentation. Moreover, each one of these characteristics is genetically complex. For example, whereas the inability of wild-type *E. coli* to utilize citrate is not accompanied by any inability to utilize other Krebs-cycle intermediates, such as α-ketoglutarate and succinate, mutation in *A. aerogenes* leading to inability to attack citrate is accompanied by the inability to attack other compounds in the Krebs cycle. Further mutation may restore some of the latter ability (65). Thus, since a single mutation will rarely produce a different species, recombination could be an important means of bringing together rapidly the constellation of genetic factors that can constitute the adaptive genotype of a new species.

The particular processes by which genetic recombination is achieved in bacteria have been thoroughly reviewed in recent times (27, 43, 67). For the purposes of the present discussion, the three principal ways will be discussed briefly in turn.

1. Conjugation. In some strains of bacteria one finds that certain cells are capable of attaching to each other, and that during the period of attachment genetic material is transferred from one cell to the other across the "conjugation bridge." This process of conjugation has been shown to involve actual cell-to-cell contact (5, 19), and the transfer of genetic material is unaffected either by the enzyme deoxyribonuclease or by antisera specific for the various kinds of viruses that infect the bacterium in question. In these respects, conjugation differs from the two other processes of genetic transfer which will be discussed below. Following the transfer of genetic material, bacteria possessing genetic material combined from both parents appear in the culture. Although conjugation has now been reported in at least five genera of bacteria (*Escherichia* (41), *Shigella* (55), *Salmonella* (9), *Serratia* (10), and *Pseudomonas* (31)), only the process occurring in *Escherichia* has been thoroughly studied. For that reason, only the picture of conjugation obtained by studying it in *Escherichia* will be described here.

In order for one bacterium to conjugate with another, it has been found that certain mating-type differences must exist between them. One type is referred to as F^+, the other as F^-. A mixture of F^- with F^- cells is infertile so far as the production of recombinant progeny is concerned. While a mixture or "cross" between F^+ and F^+ has a low fertility (possibly due to the small percentage of physiological F^- variants known to exist in a genetically F^+ population), the highest frequency of recombination occurs when F^+ cells are "crossed" with F^- cells. There is one interesting feature of the F^+ characteristic: it is generally highly infectious, appearing in all of the F^- cells with which the F^+ cells come into contact. Such a high frequency of transfer is not observed for other hereditary characters.

Another important fact which has been established concerning the transfer of genetic material occurring during conjugation is its one-way nature. If an F^+ bacterium is allowed to conjugate with an F^- bacterium, and if, after a suitable period of time is allowed for genetic transfer, the conjugating pair is physically separated by means of a micromanipulator, recombinants are found only within the clones that are produced by the F^- ex-conjugant, never within the clones produced by the F^+ ex-conjugant (4, 44). This finding is consistent with the view that genetic material passes only from F^+ to F^-, and never in the reverse direction. It is also consistent with previous observations by Hayes (28) that a number of physical and chemical agents can be used to kill the F^+ conjugant without disturbing subsequent genetic recombination; the same is not true for the F^- conjugant.

In an $F^+ \times F^-$ "cross," in which, say, the F^+ parent is $Ab\ldots$ with respect to a given pair of characters and the F^- parent is $aB\ldots$, the proportion of recombinants of a given kind, say $AB\ldots$, within the total mixed population is usually rather low (less than 10^{-4}). Wollman, Jacob, and Hayes (85) have provided several pieces of evidence which support the following explanation for this low incidence of recombinants of a specific kind. A given F^+ population is heterogeneous in containing a number of mutants, each capable of delivering at high frequency (in 100 per cent of its contacts with F^- cells) a *part* of the F^+ genome. These mutants are called "Hfr." The part of the F^+ genome that is transferred is usually large enough to consist of several genetic markers, but the particular group of

genetic markers carried depends on the particular Hfr mutant involved (36).

It is possible, by a number of ways, to show that the transfer of the particular group of markers characteristic of a given Hfr strain exhibits polarity and occurs in an ordered sequence. Everything occurs as though the particular group of markers were linked in a linear array, as though the group transferred were a segment of the complete, linearly arranged Hfr genome, as though one end of the transferred segment regularly entered the F^- recipient first, as though the segment were transferred at a uniform rate and each marker in the linear linkage group appeared in the F^- cell at a fixed time. Any experimental procedure (such as prematurely lysing the Hfr conjugant) which disturbs this orderly transfer, results in the appearance in the F^- recipient of only a fraction of the total Hfr segment that is capable of being transferred into it.

It is theoretically possible to construct a map of the complete F^+ genome by studying the linkage relationships of markers transferred by different Hfr mutants of the F^+ strain. However, there are several problems attending this task, and suffice it to say the matter is not yet resolved.

Lederberg (42) points out that the available evidence is also consistent with the view that the entire F^+ genome is transferred by an F^+ or Hfr conjugant, but that a segment of the transferred genome is eliminated from the F^- recipient prior to the formation of recombinant progeny. In this view, not all the possible recombinations of F^+ and F^- genetic material can be produced, although the F^- ex-conjugant is a complete zygote (or holozygote) rather than a partial zygote (or merozygote) as in Wollman, Jacob, and Hayes' view. Studies of the segregation and recombination of F^+ genetic material in the clones descending from the F^- ex-conjugant (4, 44) show that, indeed, not all recombinations occur nor are they reciprocal (an *AbC*. . . . for every *aBc*. . . . , for example). Results of such studies also show that either an incomplete F^+ genome is transferred or, if a complete F^+ genome is transferred, a specific part of it is lost shortly after conjugation.

Nevertheless, it must be emphasized that, although the conjugation of a given pair of F^+ (or Hfr) and F^- cells results in recombinations that are neither reciprocal nor reflect the total assortment possible from the particular F^+ and F^- genomes, conjugation in bacteria serves the same function as sexual reproduction in higher organisms. It can achieve the diffusion of genetic material through a population, and it can increase the rate of production of genetic diversity.

2. Transduction. This similarity of conjugation to sexual reproduction in higher organisms is equally true of the processes of bacterial recombination now to be discussed, transduction and transformation.

Transduction is defined as the transfer of a portion of the genome of the bacterium previously infected by a temperate bacteriophage into its new host. This phenomenon was first observed among the salmonellae using PLT22 phage (86). Here it was found that if the previously infected bacterium (the donor) was genetically *AbC*. . . . and the newly infected bacterium (the host or recipient) is *aBc*. . . . , *abc* recombinants or *ABc* recombinants or *aBC* recombinants may be produced. It is to be noted that generally only a single marker is transduced by a given salmonella phage. Yet different phage particles in a particular donor lysate carry different donor markers to the host cells they infect, so that, on a population basis, most, if not all, markers of the donor genome are transferrable to recipient bacteria.

Unlike conjugation, therefore, in which several genetic markers affecting quite distinct functions of the bacterium are generally transferred from F^+ donor to F^- recipient, transduction rarely achieves the transfer of two or more functionally unrelated markers from donor to host. Only closely linked, and usually functionally related markers are sometimes found to be transduced together (20, 52).

Since the observation of transduction depends on the temperate nature of the bacteriophage vector, a brief mention must be made of the difference between temperate and virulent bacteriophages. For a full account, see (12). When a particular bacteriophage infects a host bacterium of a particular strain, the resulting host-bacterium complex may enter upon either of two series of events. In one series of events, the so-called lytic cycle, host bacterial metabolism is radically altered to produce numerous replica phage and the eventual dissolution of the bacterial cell. In the other series of events, the so-called lysogenic cycle, the host bacterium is not radically altered in its metabolic properties although it possesses a genetic factor, called a *prophage*, which is regularly inherited by all progeny of the infected

host and which endows the cells bearing it with the capacity of producing mature, infectious phage of the type which infected the original host. This liberation of mature phage either occurs spontaneously (in one of about 10^5 lysogenized descendants) or may be induced by a variety of chemical or physical agents, such as ultraviolet light. For a given host strain, a particular bacteriophage is said to be temperate if the probability is great that infection by it will result in a lysogenic cycle; it is said to be virulent if the probability is great, on the contrary, that infection by it will result in a lytic cycle.

It should be pointed out that, although a bacteriophage must of necessity be temperate if a transduction is to be observed, lysogenization of the host bacterium is not obligatory. Transduction may or may not be accompanied by lysogenization.

There are a number of lines of evidence demonstrating that, in a lysogenized bacterium, the site where prophage replication is coordinated with bacterial reproduction happens to be a specific locus in the host bacterium's genome. Whether the prophage is attached as a small sidechain to the longer linear genome of the host or whether it constitutes an integral part of the linear structure of the host's genome is not yet resolved.

As we have noted above, in salmonellae practically any genetic marker of the donor may be transduced from donor to host, but usually different markers are borne on separate phage particles. Another type of tranduction is observed in *Escherichia coli* strain K12, where only a very few specific markers can be transferred. In this strain, bacteriophage λ transfers only the *gal* locus which affects the ability to ferment galactose. Interestingly enough, conjugation studies have shown that the site of attachment of prophage λ is very closely linked to the *gal* locus (58).

These facts suggest that in salmonellae the site of attachment of prophage PLT22 is variable and that the particular bacterial markers it will be capable of transducing are those located in the bacterial genome immediately adjacent to its point of attachment. In *E. coli* K12, on the other hand, the site of λ prophage is presumably fixed, namely, to the *gal* locus. Whether a bacteriophage is a generalized or specialized transducer (as with, respectively, PLT22 in salmonellae and λ in *E. coli* K12), seems to depend, therefore, upon whether it has fixed or variable sites of attachment in the particular bacterial strain it infects.

3. Transformation. In certain species, exposure of bacteria to the deoxyribonucleic acid (DNA) fraction extracted from mutant cells results in the genetic incorporation of determinants normally inherited by the mutants. Such genetic transfers are termed transformations. They differ from similar changes effected in transduction and conjugation in that the agent responsible for transformation is sensitive to the enzyme which depolymerizes DNA. Indeed, all of the chemical evidence points to the conclusion that these transformations in bacteria are mediated directly by DNA (32); in any event, they neither require direct contact of the host and donor cells, nor bacteriophage as an intermediary agent of transfer, nor are they prevented in the presence of proteolytic enzymes, of protein-denaturing agents, of ribonuclease, or of specific polysaccharide-combining agents.

Neither the power of bacteriophages to infect a bacterium nor their power to transfer the genes of their previous hosts is sensitive to the enzyme deoxyribonuclease. At least, this is true for bacteriophages in their extracellular form. This fact is presumably due to the protein coat which protects their internally located DNA. In any event, it seems reasonable that DNA bears the genetic information that is transferred in either transformation, transduction, or conjugation. That DNA is the principal substance of phage that enters the bacterial host is well demonstrated (29). Moreover, the transfer of genetic information in either transduction or conjugation is highly sensitive to the decay of DNA-incorporated P^{32} and to ultraviolet light (23, 24), which result is to be expected if DNA is the material that is actually transferred in these processes.

Transformations were first observed with the pneumococci (7, 26), but since that time similar processes have been found to occur among the genera *Haemophilus* (2), *Neisseria* (3, 15), *Agrobacterium* (38), *Xanthomonas* (17), and recently in *Bacillus subtilis* (78). In a transformation experiment, DNA is extracted from a donor strain genetically marked *AbC*. . . . ; an appropriate recipient strain, one marked *aBc*, is then exposed to the more or less purified donor DNA. Shortly after exposure, the recipient cells are plated on suitable selective media, on which the recombin-

ant transformed recipients, of type *ABc*. . . . etc., can be detected. As in transduction, the transformation of a given recipient bacterium rarely results in the replacement of more than one genetic determinant (*A* or *b* or *C*, in the case cited above). Nevertheless, closely linked determinants are known to be transferred together during transformation (34, 35, 69).

The process of transformation obviously has great significance for the future study of the genetics of somatic cells of higher organisms (66). Whatever information is elicited regarding the mechanism of this process should prove useful not only in extending transformation techniques to more groups of bacteria, but also in investigating the hereditary differentiation of the somatic cells of a higher plant or animal. To date we know that, in order for transformations to occur, the recipient cells must be in a physiological state of competence, which is geared to cellular reproduction and apparently involves protein synthesis (22). When competent, the bacteria can make an effective contact with transforming DNA in a very brief period of time (probably less than a minute). Having made this effective contact, the recipient bacterium is on the way to becoming transformed, which process can no longer be reversed by deoxyribonuclease. However, suitable physiological conditions and sufficient time must be provided for the expression of the phenotype corresponding to the newly acquired genotype. Furthermore, it is known that the transforming DNA acquired by the recipient bacterium is not immediately integrated into its genome. This bacterium reproduces and one or more generations may elapse before the transferred segment of donor DNA is integrated into the genome of whichever of the progeny cells happen to receive it. With integration, this segment of donor DNA is replicated in coordination with the total genome of which it is a part, its homologue having been effectively removed (34, 68, 82).

4. Multiple methods of genetic transfer. It is altogether possible that a given bacterial species may be capable of two or more methods of genetic recombination, possibly under different conditions. For example, it is clear that both conjugation and transduction can occur in certain strains of *Escherichia coli* (58). Transformation is now being studied in groups in which lysogeny is known and, therefore, in which transduction is likely (78). Undoubtedly, as further progress is made, we shall learn of more and more instances of this kind. From an evolutionary point of view, of course, the significance is obvious. The greater the opportunities for genetic exchange between organisms, the greater the speed with which adaptive genotypes can be produced in the population to which they belong.

IV. Isolating Mechanisms

A. General

The significance of recombination that has just been pointed out has a corollary. Once an adaptive genotype has been arrived at in a limiting environment, selection will make predominant those organisms possessing this genotype. However, unless there is a bar to further mutations and especially to recombinations with the genotypes of poorly adapted individuals, the adaptations will be transient ones. Put another way, the efficiency of achieving adaptation through selection is impaired if there is no mechanism for setting limits to the loss of whatever adaptive states are acquired at a given time. In higher organisms limits of this kind are imposed through isolating mechanisms which allow transfer of genetic material and recombination to occur only within certain circumscribed populations. The living universe is not a vast community of genetically interacting organisms. Rather it is divided into a large number of populations between which "gene flow" is impossible or minimized. The modern systematist trained in genetics would assign the term "biological species" to these populations.

Is there evidence that the bacterial universe is also differentiated into a number of populations whose gene pools are separated from one another; that, in other words, isolating mechanisms may operate to limit genetic recombination in bacteria?

B. In Conjugation

The F^+ factor appears to affect the bacterium in such a way as to alter its surface antigenic properties (51) and its motility (76). These effects of the F^+ factor are not surprising in view of the specificity displayed by F^+ cells in contacting F^- cells during conjugation. Several pieces of evidence indicate that the F^+ determinant is unlike other genetic factors that are transmitted during conjugation (45). F^+ shares, however, an

important property of bacteriophage: after infection it behaves as though it were an addition to the bacterial genome. As an addition, it can furthermore exist in one of two forms: in one form, it is an unintegrated part of the bacterial genome, and may be replicated either more or less rapidly than the "chromosomal" or linked genes (being infectious or lost through dilution, respectively); in its other form, it is integrated in the sense that its replication is coordinated with the replication of the host's genes, as though attached to them. In this latter form, found in Hfr bacteria, the F^+ factor is incapable of infecting other bacteria (while permitting conjugation with them) and also prevents superinfection of its host bacterium by a nonintegrated F^+ factor. For genetic factors that are additions to, rather than essential constituents of, the genome, and which may exist either in an autonomously replicating or in an integrated condition, Jacob and Wollman (37) have proposed the term "episomes."

It should be pointed out that the F^+ factor, unlike bacteriophage, has not been obtained in extracellular form, although attempts have been made to do so (48), and F^+ contagion requires direct contact between the F^+ donor and F^- recipient cells.

What is particularly interesting about the F^+ factor is the fact that there seem to be a number of different kinds. An F^+ factor making a bacterium fertile with some F^- cultures does not necessarily make it capable of conjugating with others (49). Furthermore, there is a good possibility that a single bacterium may possess more than one F^+ factor, and that one such factor may be epistatic to another. Two strains may be intersterile, and yet differ in the possession of a factor controlling ability to conjugate with a third strain (11). As a result, a large population of bacteria capable of conjugation is separated into smaller groups between which genetic transfer can occur to varying degrees, or not at all.

How large a bacterial population may be interrelated and connected by means of a system of fertility (F) factors? There is now good evidence that gene flow can spread beyond the boundaries of groups that are recognized as species by bacterial taxonomists. Luria and Burrous (55) have shown that a number of *Shigella* strains behave as F^- strains with two *E. coli* F^+ strains. As a result of these conjugations, hybrids are found which combine characters typical of *E. coli* with characters typical of *Shigella* species. Although conjugation is as efficient (up to 100 per cent) in crosses between *Shigella* F^- and *E. coli* F^+ as in crosses between *E. coli* F^- and *E. coli* F^+, the frequency of recombination is lower in the *Shigella* × *E. coli* crosses. Furthermore, some genetic determinants of the *E. coli* parent fail completely to be transmitted to the hybrid. These results suggest strongly that genetic homology between the *Shigella* and *Escherichia* strains is incomplete, as a consequence of which integration of *E. coli* genes into the recipient shigellae is not as efficient as in an "intra-specific" cross. It may be hypothesized that in the course of evolution the processes of mutation and recombination occurring in separated populations of enteric bacteria that were originally genotypically identical led to an increasing difference in the structure of the DNA in the two populations. One might expect that this difference in structure, or lack of genetic homology, will be reflected in impaired pairing between DNA molecules of the two types. If such pairing is a necessary precursor to recombination between them, decreased efficiency in this step will result in a lowered frequency of recombination. It may even be imagined that an F^+ population may be capable of conjugating with an F^- population while still unable to have its genes integrated into the genomes of the recipient bacteria, because of insufficient pairing homology between their respective DNAs. In this regard, Lederberg and Lederberg (48) have reported strains that do not produce recombinants with an *E. coli* K12 F^- culture but that do possess an infectious F^+ factor. It was not ascertained whether those strains physically conjugated with the F^- tester, while failing to produce recombinations in it.

In summary, we can indicate at least two mechanisms that may operate to limit genetic recombination in conjugation. The first of these is differences in fertility factors, which affect conjugation compatibility. The second is differences in structure of genetic material, which affect pairing compatibility and subsequent recombinations.

Luria and Burrous (55) also tried to obtain hybridization between *E. coli* F^+ (or Hfr) cultures and several different *Salmonella* strains belonging to the antigenic group E_2. These attempts failed to produce any hybrids. Recently,

however, Baron, Spilman, and Carey (9) were successful in obtaining hybrids from "crosses" between *E. coli* K12 (Hfr or F^+) and 15 of about 85 different *Salmonella* species or strains tested. The transfer investigated was that of the Lac^+ (lactose utilization) marker from the donor *E. coli* strain to the recipient *Salmonella* strain.[2] The *Salmonella* strains that succeeded in serving as F^- recipients belonged to a variety of serotypes in the Kaufmann-White schema, so that there was no apparent association between known antigenic components in *Salmonella* species and the ability to conjugate with and integrate the Lac^+ marker from *E. coli* K12. The frequency of Lac^+ hybrids was very low in the *Salmonella* populations that proved to conjugate successfully (in the order of 10^{-8} to 10^{-9}). However, when a Lac^+ hybrid was isolated and "crossed" again with a suitably marked *E. coli* K12 Hfr donor strain, the frequency of hybrids was considerably higher (in the order of 10^{-4}). Unfortunately, it is not clear at the present time whether the low frequency of hybridization in the initial cross is due to the inability of the vast majority of recipient cells to conjugate with *E. coli* Hfr or to pairing incompatibility between the genomes of the donor and recipient strains, a pairing incompatibility which is at least partially alleviated following a successful integration of donor genetic material into the recipient cell.

Indeed, in the cases of failure to hybridize *Salmonella* strains with *E. coli*, reported both by Luria and Burrous (55) and by Baron *et al.* (9), it was not determined which of the above-mentioned mechanisms, pairing incompatibility or conjugation incompatibility, resulted in the failure to obtain recombinations. It is altogether possible that other coliform organisms possessing different F factors may succeed in hybridizing with some *Salmonella* strains. In other words, only positive hybridization results can give an estimate of the extent of gene flow between populations belonging to different taxonomic species, and this estimate must of necessity be a minimal one until there is convincing evidence that all the fertility factors have been discovered (which is certainly not true today). Even within what is regarded as a single species, *Escherichia coli*, only about 80 out of 2000 strains tested behaved as either F^+ or F^- to the particular fertility factor present in the *E. coli* K12 strain employed by Lederberg and his associates (49).

It is altogether possible that conjugation incompatibility due to differences in fertility factors may be the principal mechanism serving to restrict genetic recombination between populations which are still fairly similar in their genomes and hence would be regarded as members of the same species by a taxonomist considering only phenotypic patterns. Pairing incompatibility due to major differences in DNA structure may be the principal mechanism restricting genetic recombination between populations that have evolved to a considerable extent from an originally similar genotype condition.

C. In Transduction

The ways in which transduction can be limited as a means of exchanging genetic information between bacterial populations are not difficult to visualize. Bacteriophages are fairly specific in their ability to infect bacterial hosts. Bacteriophages capable of infecting most strains within the same bacterial species are uncommon, but even less frequent are bacteriophages capable of infecting different species within the same genus, and rarest of all are bacteriophages capable of infecting different genera.

The ability of a bacteriophage to be adsorbed by and to infect a bacterium is a genetic character, and may be altered by a mutation in the phage genome or by a mutation in the bacterial genome. Thus, resistance by a bacterium to a specific bacteriophage can result either from a bacterial mutation or from a phage mutation. Conversely, the pattern of infectivity by a given bacteriophage in a number of bacterial strains (the so-called "host range" of the phage) is alterable by mutation, and by recombination as well.

It is obvious that any mutation or recombina-

[2] However, in the *Salmonella typhimurium* strain TM-9, the transfer was studied in greater detail (8). In this strain it was found that genetic factors for several different biochemical traits (including lactose utilization and indole production) could be transferred, albeit with different efficiencies. The transfer of certain other markers (including those for λ-sensitivity and for somatic and flagellar antigens) was not detected. Furthermore, the hybrids produced on using an *E. coli* F^+ donor did not appear to be infected by the F^+ agent. Similar and additional results, including hybridization of phage sensitivity and antigenic characteristics, have been reported by Miyake and Demerec (57).

tion resulting in the restriction of a bacteriophage's ability to infect will thereby limit its ability to transduce bacterial genes. For example, Lennox (52) found that the wild-type phage P1, which does not form plaques on the K12 strain of *E. coli*, does not transduce genes into this strain; however, mutants of this phage, P1 *k* and P1 *kc*, which were selected for their plaque-forming ability on the K12 strain, can transduce genes into this strain. Furthermore, mutations and recombinations in either phage or bacterial genomes may affect the relative probability of setting up a lysogenic or lytic cycle for a given bacterium-bacteriophage complex. Genetic changes leading to a higher probability of the lytic cycle (or, in other words, making the phage more virulent) will also lead to a limitation of transduction. Although lysogenization is not obligatory for transduction to be carried out, the bacteriophage must obviously be temperate for a transduction to be effected. Indeed, there is good evidence that transductions are produced by a small defective percentage of a bacteriophage population, by phages that can infect but cannot give rise by themselves to mature infectious particles (1, 14).

It is not yet settled whether all transductions, generalized as well as specialized, result from the carrying by the transducing phage of that portion of its previous host's genome to which it had been attached (12). The assumption that generalized transductions are due to phages that have variable sites of attachment to the host genome, whereas specialized transductions are due to phages that have a fixed site of attachment, is consistent with the available evidence. If this assumption is correct, moreover, it is possible to imagine another mechanism for limiting genetic transfer by transduction. Any genetic change of temperate bacteriophage or of its host that alters the number of sites to which the phage can be attached will affect the amount of genetic information it can transfer. Certainly bacteriophage λ in *E. coli* K12 is of less significance as a means of increasing bacterial recombination than is bacteriophage PLT22 in the salmonellae.

Although rare, bacteriophages are known that cross taxonomic species and genus boundaries in their infectivity. It has been shown that at least some of these bacteriophages with broad host ranges can also effect transductions between species or even genera. Lederberg and Edwards (47) demonstrated that phage PLT22, derived from a lysogenic strain of *Salmonella typhimurium*, can bring about antigenic recombinations between strains of *Salmonella* which have, on taxonomic grounds, received species status. As a result of such recombinations, several serotypes were produced that had been previously discovered in nature and classified in the Kaufmann-White schema of classification. In addition, other serotypes were produced that had so far been undiscovered in nature.

Lennox (52) has shown that phage P1, originally isolated from the Lisbon-Carrere strain of *E. coli*, and its mutant derivatives P1 *k* snd P1 *kc* are capable not only of transducing characters within and between a number of strains of *E. coli* (B/r, C, W, K12), but also of transducing characters between *E. coli* and *Shigella dysenteriae* strain Sh. Phage P1 grown on *S. dysenteriae* can transduce such characters as galactose-utilization, pyrimidine-, tryptophan-, and arginine-independence into the appropriate mutants of *E. coli* strains B, C, and W. Phage 1 *kc* grown on *E. coli* K12 can transduce such characters as arabinose-utilization and lactose-utilization into *Shigella* recipients. The Lac^+ *Shigella* strain produced by transduction still retained its other shigella characters, such as nonmotility and inability to produce indole. The Lac^+ strain of *Shigella* is, therefore, a novel bacterial strain, and is especially interesting because the Lac^- character of shigellae found in nature seems to be quite stable, spontaneous Lac^+ mutants not being found at least in the Sh strain.

Thus, hybridization can be effected between *E. coli* and *S. dysenteriae* by both conjugation and transduction, which indicates at least partial genetic homology between these organisms.

D. In Transformation

Apparently the physiological state of competence essential for transformability, which seems to involve the synthesis of DNA-adsorbing sites on the surface of the host bacterium, is nonspecific so far as the source of the DNA is concerned. DNA from a different species of bacterium is picked up as avidly by competent bacteria as DNA obtained from the same strain as that of the recipient bacteria (33, 72). Nevertheless, there may be other physiological barriers that may exclude potentially transforming DNA from entering a particular bacterium. It has

been shown that the transformability of a pneumococcus varies inversely as its production of capsular polysaccharide (68), thus suggesting that the nature of the cell wall and capsule can affect a bacterium's ability to recombine its genetic material with that of another organism by means of the latter's extracellular DNA. That some populations of bacteria regularly liberate DNA into their environment has been demonstrated. Catlin (15) has shown that certain neisseriae, upon aging, extrude DNA in their slime layer. Furthermore, she has shown that this DNA is effective in transformation.

Probably the most important barrier to genetic transformations occurring between different populations of bacteria is their degree of genetic homology, as reflected in the structure of their respective DNAs. To date a number of cases have been reported of transformations produced between populations regarded as distinct species by the bacterial taxonomist. Schaeffer first reported interspecific transformation between *Haemophilus influenzae* and *Haemophilus parainfluenzae* (75). Leidy *et al.* (50) extended this finding for a number of *H. influenzae* and *H. parainfluenzae* strains. They also reported failure to transform *Haemophilus suis* with DNA from either *H. influenzae* or *H. parainfluenzae*, although it was transformed by autologous DNA. Bracco *et al.* (13) reported transformation of two viridans strains of *Streptococcus*, using DNA either from these same strains, or from *Streptococcus salivarius*, or from pneumococci. Pakula and his associates (62) have extended the investigations on streptococci, and have reported that of 45 viridans strains tested, only 13 were transformable. Each of these 13 strains was transformed by DNA from any one or more of 5 different viridans strains, 3 *S. salivarius* strains, 1 *Streptococcus SBE* strain, 4 hemolytic streptococcus strains, and 1 enterococcus strain. None of 16 *S. salivarius* strains tested could be transformed, but since they were recently isolated from human saliva and presumably encapsulated, the failure could be due to poor penetrability of DNA into these bacteria. Of a number of hemolytic streptococci, only two strains belonging to group H were transformed; these two strains were transformed by DNA from viridans streptococci, *S. salivarius*, *S. SBE*, and heterologous hemolytic streptococci. *Streptococcus SBE* was also transformed by these DNAs. Further work by Pakula's group demonstrated that intergeneric transformations were possible as well (63). The unencapsulated R36A strain of pneumococcus was transformed by DNA from viridans streptococci, *S. salivarius*, *S. SBE*, and hemolytic streptococci. Conversely, *S. SBE*, viridans and hemolytic streptococci were transformed by pneumococcal DNA. In addition, successful transformation was reported using staphylococcal DNA on one strain of *S. SBE* and one strain of hemolytic streptococcus. Thus, the relationships of the pneumococcus-streptococcus-staphylococcus group of bacteria are susceptible of genetic analysis. Catlin (16) has also been able to perform interspecific transformations of *Neisseria* species. It is noteworthy that DNA liberated into the medium by autolyzing cells in an aging culture is as effective in inducing interspecific transformations as DNA extracted from the cells by chemical means.

In general, where quantitative measurements were made of the frequency of transformation, it has been found that for a given genetic characteristic the frequency is lower in inter- or heterospecific transformations than in intra- or homospecific transformations. Schaeffer (74) studied the question of whether the low frequency of interspecific transformations was due to the heterospecific origin of the genetic marker itself or of the entire DNA transforming molecule, of which the marker is a part. The particular marker he used was one (Sm) conferring resistance to streptomycin. To resolve the problem, he extracted DNA from a heterospecific transformant (XSmY) and tested it on the original recipient strain (X), comparing it with the DNA extracted from the original streptomycin-resistant donor strain (YSm). He found that such DNA does not behave like the original donor DNA. On the original recipient strain X it behaves like the DNA from that strain; that is, the DNA from XSmY behaves like DNA from XSm (or XSmX) rather than like DNA from YSm (or YSmY), in its efficiency of transformation. Thus, in the course of transforming bacteria with DNA from a different species, the transformant itself is relieved of whatever factors contributed to the lower efficiency of interspecific transformation. That the DNA of XSmY does retain some of the Y-specific structures, however, is suggested by the fact that higher frequencies of transformation are obtained in the reaction Y $\times$ DNA of XSmY than in the reaction Y $\times$ DNA of XSmX.

These results are consistent with a hypoth-

esis according to which pairing between the endogenous (or host bacterium's) DNA and the exogenous (transforming) DNA is a necessary prelude to integration of a particular exogenous marker (74). It is assumed that transformation does not necessarily involve integration of the entire molecule bearing the marker in question, which is supported by the results of linked transformations. It is further assumed that the exactness of pairing depends on the structural homology of the endogenous and exogenous DNA molecules: the less complete the homology, the less exact is the pairing, which results in a lowered efficiency of integration of an exogenous marker into the recipient genome. It will be noted that this hypothesis is essentially the same as the one proposed to account for lowered recombination frequencies in interspecific conjugations (see above). In the case of heterospecific transformations, moreover, it appears that the integrated marker may be freed from those adjacent regions of the exogenous molecule which caused a lowered probability of integration.

Schaeffer (72, 73) then demonstrated, by means of DNA labeled with P^{32}, that DNA from one species of *Haemophilus* can be taken up by bacteria of another *Haemophilus* species with the same ease as DNA of the same species. The same number of DNA molecules, as measured by P^{32} uptake, is incorporated per competent cell in either case. This follows from the fact that the DNA from a number of different species (although not all) have the same affinity for the surface receptor of the host strain, and indeed compete with each other for penetration into the cell when presented as a mixture to the recipient bacteria. Since DNA of, say, *H. parainfluenzae* has no difficulty getting into *H. influenzae*, then the fact that the frequency of such interspecific transformations is lower than the intraspecific ones must be due to some process occurring after penetration. This finding further supports the hypothesis of inexact pairing.

Does such pairing incompatibility arise only when bacterial populations have evolved genetically to a considerable extent, so that they are recognized as different species? Recent evidence indicates that pairing incompatibility may arise within very closely related strains. Green has studied (25) the frequency of transformation by two different markers in two unencapsulated strains of pneumococcus, Rx and Rz, which were independently isolated although closely related in heritage (68). The two markers were ery_2 and *str*, which confer resistance to erythromycin and streptomycin, respectively. It was observed that, at any DNA concentration, the ery_2 marker transforms both strains with equal frequency, but the *str* marker transforms the Rz strain with a fourfold less efficiency than it does the Rx strain. Thus, there appears to be a specific effect depressing the frequency of *str* transformations in the Rz host strain. Green showed that, by varying conditions under which the doubly marked DNA was adsorbed to the Rz strain, he could alter the frequency of transformation by both markers by as much as a thousandfold, but the ratio of ery_2 to *str* transformations in the Rz strain was independent of these changes. It did not appear that selective nonadsorption of the *str* marker by the Rz strain could explain the lowered frequency of *str* transformations. As a matter of fact, by examining the frequency of transformants (recipient cells that adsorbed, integrated, and phenotypically expressed the acquisition of the marker) at various times after the recipient cells made contact with the transforming DNA, Green found that the initial rate of appearance of *str* transformants was the same in the Rx and Rz strains. At about 45 minutes after contact, while *str* transformants continued to be produced in the Rx strain, no further *str* transformants were produced in the Rz strain. It appears as though some postinfection process, necessary for the completion of transformation, is terminated earlier in the Rz strain than in the Rx strain. It is possible to imagine again that pairing difficulties specific to the *str* region in the Rz host lead to a premature termination of the integration of the *str* marker in that strain.

The question arises as to whether the factor causing a depressed frequency of *str* transformations is lost when the *str* marker is successfully integrated into the Rz host, as was found in the case of the interspecific *Haemophilus* transformations studied by Schaeffer. Green investigated this possibility by isolating 21 different *str* transformants produced in the Rz strain by a DNA yielding *str* transformations at a depressed frequency. Each *str* transformant was the result of a separate transformation of a recipient bacterium. These transformants were then individually transformed by the ery_2 marker. The DNA was then extracted from each of these doubly marked strains, and was tested on both the Rx and Rz strains. All 21 DNAs produced

ery$_2$ and *str* transformations with equal frequency in the Rx strain. However, only one of these DNA preparations produced this result in the Rz strain. Twenty of these preparations yielded *str* transformations at the previously observed, fourfold depressed frequency. Thus, unlike the situation in interspecific *Haemophilus* transformations, the *str* marker was found to be transferred, in most cases, along with the "depressing factor." The latter, while separable from the *str* marker, was closely linked to it, and behaved like a transferable genetic factor.

Green (25) also showed that ultraviolet irradiation has an interesting effect on the doubly marked DNA preparation from which the *str* marker is transferred into the Rz strain at a depressed frequency. When heavily irradiated, such DNA continues to yield *str* transformants at a depressed frequency, but the DNA subsequently extracted from these *str* transformants is often found to be relieved of the "depressing factor." Seventeen such transformants were examined, by first transforming them with the *ery*$_2$ marker and then extracting DNA from them. These DNAs were then compared on the Rx and Rz strains. Fourteen were found to be relieved of the "depressing factor," transferring the *str* marker at a normal frequency (relative to the *ery*$_2$ marker), while three continued to reveal the presence of the "depressing factor." Thus, ultraviolet light appears either to destroy selectively the "depressing factor" or to increase the probability of recombination away from it.

In any event, Green's work shows that DNA incompatibility factors can be found within very closely related populations. It suggests that such factors may be the raw materials from which stronger barriers to interspecific transformations may be constructed.

V. Bacterial Speciation

The recent investigations of recombination in bacteria have afforded an excellent opportunity to study the genetic relationships of different bacterial populations. A particular result of these investigations, which it has been the burden of this article to demonstrate, is that for each mode of genetic recombination (conjugation, transduction, or transformation) specific isolating mechanisms exist. Genetic recombination can, indeed, be severely limited between populations of bacteria capable of the same mode of recombination.

The question arises, therefore, whether these isolating mechanisms separate only populations belonging to what the bacterial taxonomist has called species, and are lacking in populations belonging to the same taxonomic species. In other words, the question is really the one posed at the outset of this review: whether the species of the taxonomist correspond exactly to species defined on the basis of genetic recombination. Numerous instances have been cited in the preceding sections that exact correspondence is not observed.

Before proceeding to discuss why this finding should not surprise us, it is perhaps useful to recall what van Niel (84) has so well stated: the tasks of the classifier and of the student of genetic relationships are not to be confused. The construction of a practical key for the classification of the bacteria, which has been the primary goal of the bacterial taxonomist up to the present time, is a necessary and important task. The sole criterion for the adoption of a particular key should be the empirical one of whether it works, whether one finds the designation for the organism one is studying. Other criteria must be used, however, when schemas are proposed for the probable evolutionary relationships of the bacteria. Here we must have evidence for asserting ". . . that group A is more closely related to group B than it is to group C because the separation in descent of group A from group B is more recent than the separation of groups A and B from group C." It is precisely in questions of this sort that genetic evidence is important. In discussing bacterial evolution, moreover, it behooves us to utilize a definition of bacterial species that reflects the mechanisms involved in speciation.

In the preceding sections a model was presented for the consequences to genetic material of evolutionary change. As a bacterial population evolves, the predominant form of its genetic material is changing. Mutation and presumably recombination bring forth new genotypes, and natural selection causes the most adaptive of these to prevail at any given time. Since DNA is a major component of the genetic material, one can expect that these processes will result in differentiation or divergence of the evolving DNA from that of the ancestral type. Lanni ((39) and *personal communication*) has recently performed an important scholarly job in bringing together the available evidence concerning the ways in

which DNAs of a wide variety of organisms differ. The evidence he has compiled, which is admittedly fragmentary, points suggestively to the conclusion that differentiation proceeds by way of alteration of the relative proportion of adenine + thymine (A + T) to guanine + cytosine (G + C) nucleotides in the DNA molecule. Groups of bacteria that are known on other grounds to be closely related generally possess DNAs having similar (A + T)/(G + C) ratios. Thus, for example, this ratio for *E. coli*, salmonella and shigella DNAs is about 1, while *Aerobacter aerogenes* and *Serratia marcescens* have DNAs with a ratio of 0.76. *Proteus* species, on the other hand, have a DNA with an (A + T)/(G + C) ratio of 1.75.[3]

At what point in the divergence of a bacterial population from some ancestral type is it to be regarded as a distinct species? In answering this question, one must first point out that if genetic divergence alone occurred in nature, the taxonomist would be unable to recognize discrete populations of bacteria possessing characteristics that clearly distinguished one from another. Genetic divergence alone would make for a broad, extreme form of heterogeneity lacking in any discontinuity which would permit the human observer even to conceive of taxonomic categories. Yet there is sufficient discontinuity in the bacterial universe to permit a fairly complex system of classification in which the various orders and families are quite distinct (80, 84). Distinctness of boundaries falls off, on the other hand, at lower taxonomic levels. Especially as one examines samples from similar ecological niches collected at different places, populations are found which show some degree of "overlapping," and "intermediacy" is common. It is probably true, however, that in a particular local or micro-environment bacterial populations are more sharply delineated from each other, and intermediate types are a less common occurrence.

To what is this discontinuity of genetic types due? Assuming that the modes of recombination described above operate in natural bacterial populations, it becomes obvious that the acquisition of isolating mechanisms would serve to limit the amount of genetic divergence obtainable from recombination. The existence of a mechanism preventing "gene flow" between two bacterial populations could then serve as the criterion for calling them distinct species. Yet we have already noted that the taxonomist's species do not always correspond to those populations which are completely incapable of exchanging genetic information, as evidenced by an experimental test of recombination. The reason could easily stem from the very nature of evolution itself. Evolution is a dynamic process, and speciation is sometimes unwittingly observed in transition. Two populations that are diverging may not yet have reached a stage of complete isolation, and it is still largely an arbitrary matter deciding whether the differences between the two populations are sufficient to warrant their designation as separate species, at least from a taxonomic point of view.

Furthermore, it needs to be understood that isolating mechanisms differ and may possess intermediate degrees of efficiency as well as absolute ones. Consider the circumstances under which bacterial evolution may occur. We may visualize a part (A′) of some bacterial population (A) exploring a new ecological niche for which it is still poorly adapted. If no competitor species are present in this niche, or, if present, they are not completely exhausting the possibilities for further expansion in the niche, evolution of an adapted form of A′, or B, will arise in time through the gradual action of mutation, recombination and selection. Two possibilities may exist, however, so far as the spatial separation of B and A are concerned:

Possibility I: The nature of B's ecological niche does not assure complete separation of B from its ancestral population A, or, for that matter, from an evolutionarily related population (*i.e.*, another population also derived from A).

a. If in the course of evolving the adaptive

[3] An extraordinary finding of recent studies using density-gradient centrifugation has been the high degree of homogeneity in the population of DNA molecules from a given species (70, 81). In investigations of the relation between buoyant density in a cesium chloride solution and the base composition of DNA, several bacterial species were used in which the fraction of guanine-cytosine in their respective DNAs varied from 30 to 70 per cent. It was found that the buoyant density was directly proportional to the guanine-cytosine content. But more remarkable still, in the words of Rolfe and Meselson (70), was the fact ". . . that the standard deviation of guanine-cytosine content within the molecular population of any one bacterial species covers less than one-tenth of the range over which the mean guanine-cytosine content varies among the various species."

genotype characteristic of B, the change in DNA structure is great enough, pairing incompatibility will assure genetic isolation. This may be the way in which certain strains of streptococci and pneumococci, which inhabit very similar, if not adjoining niches (in the respiratory tract of man and other animals), have become incapable of recombining with each other.

b. If the change in DNA structure is not very extensive, so that pairing incompatibility is slight, other isolating mechanisms may be selected for until such incompatibility develops. This may be the way in which different strains of *E. coli*, with presumably slightly different ecological preferences, are genetically isolated from each other. Differences in the system of fertility factors affecting conjugation could account for such isolation.

Possibility II: The nature of B's ecological niche assures a very low or negligible probability of contact by B with its ancestral species A. In this case, the ecological isolation itself serves to prevent genetic recombination between the two populations. No mechanism preventing recombination may ever arise unless the divergence in the respective DNA structures is great enough to assure complete incompatibility in the event (accidental or experimental) that the two populations, or subclones of them, ever confronted each other. This may be the way in which two parasitic populations, which lie in different parts of the host's body may be isolated from each other.

Before testing these ideas, however, we first need to know if the modes of genetic recombination that are known to occur *in vitro* actually occur in nature. We know that certain bacteria are potentially capable of recombination, but it is not yet certain whether recombination is utilized in nature at all or whether the extent to which it is utilized has any evolutionary force. Until we can be sure of the extensive utilization of the modes of recombination available to the bacteria, we do not know if the isolating mechanisms that have been discussed above have any evolutionary significance. *A priori*, however, there seems little reason to doubt that recombination occurs to a significant extent among bacteria in their natural environments. Austrian (6) has demonstrated that transformations of pneumococci can occur in the bodies of a number of animal species, and that such transformations result in recombination of genetic factors affecting capsule synthesis and somatic antigens. Moreover, the frequencies of recombination that are now observed *in vitro* are appreciable. Conjugation in *E. coli* is nearly 100 per cent efficient, and recombination of genes between a specific Hfr donor and an F^- recipient can be as high as 20 per cent. Transformation frequencies are sometimes as high as 10 to 25 per cent.[4]

If, on the other hand, genetic recombination does not occur to any significant extent in nature, and represents only an experimental trick of recombining bacterial genes in the bacteriologist's test tube, we are then forced to consider the meaning of the *capacity* for recombination that bacteria possess. It is conceivable that recombination is an inherent property of the genetic apparatus of bacteria, by virtue of the chemical nature and organization of bacterial genes, and that this property is expressed only among the more advanced forms of life which utilized it in their elaboration of mechanisms of sexual reproduction. In this view, recombination is a latent property of the genetic material of the more primitive forms of life, a pre-adaptation, so to speak, for the evolutionarily more advanced forms that followed. Another possibility, suggested by R. C. Lewontin (*personal communication*) is that the *capacity* for recombination in bacteria is an evolutionary relic. In this view, the bacteria descended from primitive forms of life that included genetic recombination in their process of reproduction, as the "higher" plants and animals do today in sexual reproduction, and sex was discarded in certain lines of evolution, including the ones leading to the bacteria.

Should further study prove that genetic recombination has little or no evolutionary significance in natural bacterial populations, it will mean that mutation and selection are the

[4] The frequency of conjugation or transformation does depend on environmental conditions. In this regard Lewontin (53) has pointed out that in animals whose life cycles consist of alternating asexual and sexual phases, the sexual phase is often correlated with the appearance of unfavorable physiological conditions in the environment. The significance of saving recombination for environmental exigencies is obvious. It would be interesting to see if recombination among bacteria in nature is also induced by unfavorable environmental conditions.

primary forces permitting bacterial evolution. In order to account for the discontinuity of genetic types that we recognize among the bacteria, we would have to suppose that periods of greatly relaxed selection occur from time to time in the bacterial world. Only under such circumstances could mutations accumulate to bring about new types diverging from the original types in a sufficiently complex and multiple way. Then, when changing environmental conditions make selection more rigorous, bacteria with nonadaptive genotypes will be weeded out. If the results of these evolutionary forces are divergences in DNA structure, the *capacity* for genetic recombination can still be used experimentally as a means of testing the degree of divergence. The extent to which recombination is experimentally possible between two DNA structures would be an index of the evolutionary relationship of the bacteria containing them. While perhaps not themselves forces directing evolution, mechanisms barring recombination *in vitro* could nevertheless provide a criterion for species distinctions.

Though the problems posed here are certainly large and difficult, the means for studying them are not altogether out of reach. There are many questions that can be posed for experimental attack. For example, it would be valuable to isolate cultures of the two related "species" from the same or adjoining ecological niches in nature (say, a pneumococcal and a streptococcal strain from the throat swab of some animal). Such freshly isolated strains can be multiply marked genetically, and one can determine if recombinations occur *in vitro*. A positive outcome would at least imply that two groups likely to confront each other in nature are capable of recombining genetic information. To determine if this were indeed occurring, the two properly marked strains could be re-introduced into the kind of ecological niche from which they were drawn, and the production of recombinants could be checked. The same kind of experiment could be performed with strains obtained from different but similar ecological niches (say, the intestinal tracts of the dog and the horse).

Other questions need answering. Why is it that of all the *Salmonella* serotypic combinations that are possible, only a fraction have been reported (although new ones are reported from time to time)? Are only certain antigenic combinations possible because they are the consequences of the only adaptive genotypes that exist? Are the bearers of certain antigenic combinations strongly selected against, or is the production of such antigenic combinations unlikely because the only parental populations that could recombine to give rise to these combinations are isolated in some way in nature?

Why is it that *Shigella* Lac^+ strains have not been observed in nature although they are readily produced *in vitro* by conjugation with *E. coli*, which is an enteric species that shigellae are likely to confront in nature? Does selection act strongly against such hybrids? Or are there mechanisms which genetically isolate these species of which we are still ignorant?

It is clear that more extensive genetic studies of an ecological nature must be carried out. Very few genetic experiments have been conducted utilizing the natural ecological niches inhabited by the bacteria being investigated. With a resurgence of interest in bacterial ecology, reinforced by our new genetic knowledge and biochemical techniques, a unification of our knowledge of the bacteria is likely to be forthcoming.

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